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Human Cytomegalovirus UL44 Concentrates at the Periphery of Replication Compartments, the Site of Viral DNA Synthesis

Blair L Strang[1], Steeve Boulant[2], Lynne Chang[3], David M Knipe[3], Tomas Kirchhausen[2], and Donald M Coen[1]*

[1]Department of Biological Chemistry and Molecular Pharmacology, [2] Department of Cell Biology, Harvard Medical School, Immune Disease Institute and Program on Cellular and Molecular Medicine at Childrens Hospital and [3] Department of Microbiology and Immunobiology, Harvard Medical School, Boston, MA 02115, USA

*To whom correspondence may be addressed;
Donald M Coen, Phone 617-432-1691, Fax 617-432-3833
E-mail: don_coen@hms.harvard.edu

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ABSTRACT

Formation of replication compartments, the sub-nuclear structures in which the viral DNA genome is replicated, is a hallmark of herpesvirus infections. The localization of proteins and viral DNA within human cytomegalovirus replication compartments is not well characterized. Immunofluorescence analysis demonstrated the accumulation of the viral DNA polymerase subunit UL44 at the periphery of replication compartments and the presence of different populations of UL44 in infected cells. In contrast, the viral single-stranded DNA binding protein UL57 was distributed throughout replication compartments. Using “click chemistry” to detect 5-ethynyl-2'-deoxyuridine (EdU) incorporation into replicating viral DNA and pulse-chase protocols, we found that viral DNA synthesis occurs at the periphery of replication compartments, and that replicated viral DNA subsequently localizes to the interior of replication compartments. The interiors of replication compartments also contain regions in which UL44 and EdU labeled DNA are absent. Treatment of cells with a viral DNA polymerase inhibitor reversibly caused dispersal of both UL44 and EdU labeled viral DNA from replication compartments, indicating that ongoing viral DNA synthesis is necessary to maintain the organization of replication compartments. Our results reveal previously unappreciated complexity in organization of human cytomegalovirus replication compartments.

INTRODUCTION

Replication of viral genomes takes place in discrete sites within the cell, which enables viruses to concentrate and organize factors required for genome replication. During herpesvirus infection, drastic and dynamic reorganization of the nucleus is observed, including the partitioning of host cell chromatin and rearrangement of cellular nuclear proteins primarily due to the development of viral replication compartments (20, 23, 26).

The formation of human cytomegalovirus (HCMV) replication compartments in infected cells has been observed, as has the localization of several viral proteins within them (2, 10, 21). It is unclear how viral proteins are organized within replication compartments and unknown where viral DNA synthesis occurs within compartments.

In a previous report from our laboratory we assayed the localization of the presumptive viral DNA polymerase processivity subunit UL44 (also known as ICP36) in infected cells as a marker for infected cell nuclei (10). Although we did not comment upon it at the time, we observed that UL44 accumulates at the periphery of replication compartments. To our knowledge, no viral protein in any herpesvirus replication compartment had shown this distribution, so we wished to investigate this observation further, hypothesizing that it might signal how DNA synthesis is organized within replication compartments. We therefore examined the localization of UL44, another viral DNA replication protein and viral DNA synthesis within replication compartments.

MATERIALS AND METHODS

Cells and viruses. Human foreskin fibroblast (HFF) cells (American Type Culture Collection (no. CRL-1684)) were used in all experiments. HCMV laboratory strain AD169 was used. Virus expressing FLAG tagged UL44 (HCMV-FLAG44) has been described elsewhere (28).

Immunofluorescence. HFF cells ($5x10^4$) were plated on glass coverslips. Cells were mock infected or infected in the presence or absence of phosphonoformic acid (520 μM) with AD169 or HCMV-FLAG44 (28) (MOI 3). Cells were fixed at room temperature with 4% formaldehyde in Dulbecco's phosphate-buffered saline (DPBS) at the time points indicated in the text. Where indicated, cells were incubated prior to fixation with 10 μM 5-ethynyl-2'-deoxyuridine (EdU) (Invitrogen) or 200 μM thymidine. Also, where indicated 520 μM phosphoformic acid (PFA) (SIGMA) was added. When necessary EdU and PFA were washed out of cells by rinsing cells 3 times with tissue culture media that did not contain either molecule. Following fixation, cells were washed with DPBS and permeabilized at room temperature (RT) for 10 min with 0.5% Triton X-100 dissolved in DPBS. Where indicated EdU incorporated into DNA was detected using “click chemistry” (25) with a fluorescent azide (Alexa Fluor 488) (Invitrogen) as per the manufacturer’s instructions (Invitrogen). Once rinsed again with DPBS, cells were incubated in 0.5% BSA dissolved in DPBS for 20 min at RT. Primary antibodies in 0.5% BSA dissolved in DPBS were applied and incubated for 1 h at

37°C. Antiserum was removed by rinsing cells once in 0.5% Tween dissolved in DPBS once and twice with DPBS, each time for 5 min with rocking. This procedure was repeated for the secondary antibodies. Where indicated, coverslips were incubated in DPBS containing 10 μg/ml Hoechst 33342 for 5 minutes before mounting. Coverslips were mounted on microscope slides with ProLong Antifade (Invitrogen-Molecular Probes), and imaged using either deconvolution microscopy or spinning disk confocal microscopy..

For deconvolution microscopy, cells were imaged on an Axioplan 2 microscope (Zeiss) with a 63× objective and Hamamatsu CCD camera (model C4742-95). Images were deconvolved using the inverse filter algorithm in the Axiovision (Rel.4.5) software.

For spinning disk confocal microscopy, images were acquired using an inverted spinning disk confocal microscope based on an Axiovert 200M inverted microscope (Carl Zeiss, Inc.; Thornwood, NY), a CSU-X1 spinning disk confocal unit (Yokogawa Electric Corporation; Tokyo, Japan), a spherical aberration correction device (SAC, Infinity Photo-Optical; Boulder, CO) and a 63X objective lens (Plan-Apochromat, NA 1.4, Carl Zeiss). Images shown were obtained by acquiring sequential optical planes of the entire cell spaced by 0.15 μm in the z-axis and projecting those planes using Slidebook 4.2 (Intelligent Imaging).

Primary antibodies recognizing UL44 (αICP36 (Virusys, CA006), CH16 (22) (Virusys) and 28-21 (3) (a kind gift from Bill Britt, University of Alabama)), , murine MAb recognizing UL57 (Virusys) and rabbit polyclonal antibody F7425 recognizing FLAG (SIGMA) were used at a dilution of 1:100. All fluorescently

labeled secondary antibodies (Alexa Fluor 488 or Alexa Fluor 594) were obtained from Molecular Probes and used at a dilution of 1:1000.

In each experiment 3-5 cells representative of the phenotypes observed throughout each coverslip ($5x10^4$ cells) were imaged and analyzed in detail. Each experiment was performed at least twice.

123 **RESULTS**

124

125 **Localization of UL44 in the infected cell nucleus.** Our laboratory has
126 previously used UL44 as a marker for infected cell nuclei in studies of nuclear
127 lamina in HCMV-infected cells (10). Unexpectedly, in these studies UL44
128 appeared to localize to the periphery of replication compartments (10). To
129 explore this observation further, we mock-infected or infected HFF cells with
130 HCMV strain AD169, and stained the cells with a monoclonal antibody (MAb)
131 recognizing UL44 (⟨ICP36) for analysis by immunofluorescence (IF). We also
132 stained cells with Hoechst stain to visualize host cell chromatin. Cells were
133 imaged using deconvolution microscopy. Single deconvolved sections are shown
134 in Figure 1A. In mock-infected cells, host cell chromatin could be observed
135 throughout the nucleus, and no staining of UL44 was observed (Fig. 1A(i)-(iii)). In
136 infected cells, at 48 h.p.i. and 96 h.p.i. (Fig. 1A(iv)-(ix)), UL44 appeared to
137 accumulate in a ring-like distribution at the periphery of viral replication
138 compartments. Only faint staining of UL44 was observed within the interior of
139 viral replication compartments, and host cell chromatin was excluded from these
140 compartments. We observed some marginalization of host cell chromatin in the
141 infected cell nucleus reminiscent of host cell chromatin partitioning seen in
142 herpes simplex virus (HSV) infected cells (20) (indicated with arrows in Fig. 1A).
143 The number of UL44-positive compartments at each time point was calculated
144 (Fig. 1B). Typically, infected cells contained 2-3 compartments at 48 hpi. By 96
145 hpi, most cells contained only one large compartment. The change in the number

of replication compartments over time may represent coalescence of replication compartments as infection progresses, as has been reported for HSV (4, 27, 30).

Different populations of UL44 in infected cells. Staining of HCMV-infected cells with MAb αICP36 (Fig. 1) indicated that UL44 is found predominantly at the periphery of replication compartments with little protein inside or outside of replication compartments in infected cell nuclei. This distribution was similar to the staining of UL44 at the periphery of replication compartments seen by in our previous report using MAb 28-21 (10). In contrast, Penfold and Mocarski observed high levels of UL44 within the interior of replication compartments when staining infected cells with MAb CH16 (21). To better understand UL44 distribution, cells were infected with AD169 or a virus expressing FLAG tagged UL44 (HCMV-FLAG44) (28), and stained with a panel of UL44 MAbs or a rabbit polycolonal antibody recognizing FLAG (Fig. 2). Cells were imaged using a spinning disk confocal microscope.

AD169 infected cells stained with MAb αICP36 showed similar phenotypes to those seen in Figure 1 (results not shown). In AD169 infected cells stained with MAb 28-21 (3) (Fig. 2D), UL44 accumulated at the periphery of replication compartments, and faint UL44 staining was detected inside and outside compartments, similar to the distribution of UL44 in AD169 infected cells stained with MAb αICP36 (Figure 1). In HCMV-FLAG44 infected cells, using a rabbit polyclonal antibody against the FLAG epitope, we observed a distribution of staining similar to that found using MAb 28-21 (Fig. 2F). In AD169 infected cells

stained with MAb CH16 (22), we again found UL44 concentrated at the periphery of replication compartments; however, notable staining was also observed within the interior of replication compartments and throughout the cytoplasm (Fig. 2E). This pattern was similar to that observed by Penfold and Mocarski (21) and further confirms that UL44 accumulates at the periphery of replication compartments. The data suggested that a population of UL44 recognized by MAb CH16 is present within the interior of compartments. Also, in cells stained with MAb CH16, we found regions in the interior of the replication compartment where UL44 could not be observed (an example is indicated with an arrow in Fig. 2E). With all of the antibodies, we observed similar results analyzing cells at 48 h.p.i. (data not shown) or at 96 h.p.i.. No obvious staining was observed at 0 h.p.i. in each case (Fig. 2A-C).

In summary, our results raise the possibility of different populations of UL44 present within the nucleus; at least one at the periphery of replication compartments and at least one that is within the interior of replication compartments. We do not know the molecular basis for the differences in staining patterns of the different antibodies. Regardless, these findings and our inability to detect UL44 in certain regions of the interior of replication compartments suggest the presence of multiple subcompartments within HCMV replication compartments.

192 **Visualization of UL44 and UL57 within viral replication compartments.**
193 We next wished to compare the localization of UL44 with that of another viral
194 DNA replication protein. We therefore assayed the localization of the HCMV
195 ssDNA binding protein UL57 relative to FLAG-tagged UL44. We infected cells
196 with a virus expressing FLAG tagged UL44 (HCMV-FLAG44) (28), stained the
197 cells with a murine MAb recognizing UL57 and a rabbit polyclonal antibody
198 recognizing FLAG (Fig. 3), and analyzed the IF image using spinning disk
199 confocal microscopy. At 96 h.p.i. FLAG tagged UL44 concentrated at the
200 periphery of replication compartments and was more diffusely distributed
201 throughout the nucleus (Fig. 3(iv)). In contrast, UL57 was observed in punctate
202 structures throughout replication compartments (Fig. 3(v)), reminiscent of the
203 staining for ICP8 observed in HSV infected cells (5, 23). No obvious co-
204 localization of FLAG tagged UL44 and UL57 was observed in the interior of
205 replication compartments; however, a few punctate areas of co-localization could
206 be seen at the periphery. (Fig. 3(vi)). Similar results were obtained analyzing
207 cells at 48 h.p.i. (data not shown). Because UL57 can bind to the viral genome,
208 these results suggested that viral DNA is present both at the periphery of the
209 replication compartment, where at least one form of UL44 concentrates, and in
210 the interior of the replication compartment.
211
212 **Visualization of viral DNA within viral replication compartments.** We next
213 sought to understand the localization of viral DNA and UL44 in viral replication
214 compartments by labeling viral DNA. A previous report (21) indicated that

bromodeoxyuridine (BrdU)-labeled DNA is present within replication compartments and throughout the nucleus of HCMV infected cells. In our preliminary experiments labeling of HCMV infected HFF cells with BrdU was very inefficient (data not shown). Instead, we assayed the localization of viral DNA by IF using 5-ethynyl-2'-deoxyuridine (EdU) (Fig. 4A). As described elsewhere (25), EdU incorporated into replicating DNA is recognized by a fluorescent azide via a Cu(I)-catalyzed [3 + 2] cycloaddition reaction ("click" chemistry).

We incubated AD169 infected HFF cells with EdU for 1 hour at 0 h.p.i. and 96 h.p.i., then prepared the cells for IF by performing the cycloaddition reaction to detect incorporated Edu and staining with MAb ⟨ICP36 to recognize UL44. Images were obtained at 96 h.p.i. using spinning disk confocal microscopy. UL44 again accumulated at the periphery of replication compartments, with faint UL44 staining in the interior of the compartment and throughout the nucleus (Fig. 4A(iv)). Punctate EdU staining was seen mainly in the interior of the viral replication compartment (Fig. 4A(v)). We also observed regions within viral replication compartments where neither UL44 nor EdU labeled viral DNA was found (white arrow in Fig. 4A(vi)), which may represent subcompartments in the interior of replication compartments. Nevertheless, co-localization of EdU and UL44 was evident at the periphery of replication compartments (Fig. 4A((vi)). Similar results were obtained analyzing cells at 48 h.p.i. (results not shown and below).

To investigate where viral DNA synthesis occurred within replication compartments, we performed a pulse-chase experiment at 48 h.p.i. (Fig. 4B). We

pulse labeled HCMV infected HFF cells 48 h.p.i. with EdU for 30 mins (the minimum period of labeling that consistently gave a signal (results not shown), and then chased with thymidine for 30 mins to stop EdU labeling. After the 30 min pulse with EdU, we observed incorporation of EdU into DNA predominantly at the periphery of replication compartments, co-localizing with UL44 detected by MAb αICP36 (Fig. 4B(i)). When EdU was chased with thymidine, DNA containing EdU accumulated within the interior of the compartments, and punctate staining of EdU at the periphery of replication compartments was less evident (Fig. 4B(ii)). Similar results were obtained analyzing cells at 96.h.p.i. (results not shown).

In sum, we found that viral DNA synthesis occurs at the periphery of viral replication compartments and then replicated viral DNA is found in the interior of the compartments.

Localization of UL44 and EdU labeled DNA in the presence of an inhibitor of viral DNA synthesis. We next sought to determine if active viral DNA synthesis plays a role in organization of viral replication compartments once the compartment is formed. We therefore labeled infected cells with EdU at 48 h.p.i. for 1 hour. After incubation with EdU, infected cells were left untreated or treated with an inhibitor of viral DNA synthesis (phosphonoformic acid, PFA) in the presence of EdU. Cells were collected at different times after PFA and EdU treatment (Figs. 5(iv)-(xii)). After 90 minutes, the PFA was removed and washed out and cells were collected at different times in the presence of EdU (Figs. 3(xiii)-(xx)).

In the absence of PFA, we observed a distribution of UL44 and EdU similar to what we had already observed (panels i - iii). Treatment with PFA caused dispersal of UL44 and EdU labeled viral DNA from replication compartments that was quite pronounced by 90 minutes (Figs. 5(iv)-(xii)). Also, by 90 minutes EdU staining was notably less bright, most likely due to lack of incorporation of EdU in the absence of viral DNA replication. When PFA was washed from the cells (Figs. 5(xiii)-(xx)), EdU labeled DNA co-localized with punctate UL44 staining within 30 minutes (Figs. 5(xiii)-(xv)). Over time the integrity of replication compartments was re-established and UL44 again concentrated at the periphery of large replication compartments that contained EdU labeled DNA (Figs. 5(xvi)-(xxi)).

These results indicated that viral DNA synthesis is required for maintaining the organization of HCMV replication compartments. Also, the appearance of large replication compartments from small punctate bodies may represent coalescence of smaller replication compartments to form larger replication compartments, as also suggested by the data in Figure 1.

DISCUSSION

Here we provide insights into the organization of HCMV replication compartments, demonstrating that viral DNA synthesis occurs at the periphery of each compartment within a layer in which the viral DNA polymerase subunit, UL44, concentrates. The viral single stranded DNA binding protein, UL57, distributes both at the periphery of the replication compartment and in its interior. The interior of the compartment also contains a population of UL44 and areas where replicated viral DNA can be found, and regions where neither UL44 nor viral DNA were detected. Thus, there are sub-compartments present within replication compartments. Viral DNA synthesis appears to be required to maintain the distribution of UL44 and viral DNA within replication compartments.

As viral DNA synthesis takes place at the periphery of replication compartments, UL44 and UL57 in the interior of compartments are most likely not directly involved in viral DNA synthesis. HSV replication compartments are the sites of transcription of late HSV genes (13, 24). The HSV counterpart of UL57, ICP8, is important for regulation of late HSV gene transcription (7, 8, 18). Additionally, it has been reported that UL44 has a role in transcription of late HCMV genes (11, 12). Therefore, UL57 and UL44 within the interior of replication compartments may be involved in late viral transcription.

Our findings that EdU labeled viral DNA is found first at the periphery of replication compartments and then in the interior of compartments in pulse-chase experiments suggest that there is a mechanism facilitating movement of

304 replicated viral genomes within the compartment and/or the movement of the
305 periphery of the compartment away from replicated genomes. What this
306 mechanism is remains unknown. However, packaging of HSV viral DNA into
307 virus capsids occurs within replication compartments (19). Thus, we speculate
308 that packaging of viral DNA may facilitate movement of viral DNA into the interior
309 of replication compartments. It is possible that the regions within replication
310 compartments where no UL44 or EdU labeled DNA could be observed may be
311 sites of capsid assembly. The encapsidation of EdU labeled viral DNA at these
312 sites may prevent detection of EdU in our analysis. The factors determining
313 efficient packaging of viral DNA are still under investigation, but could involve
314 UL44. TRS1, a protein that we have previously found to associate with UL44 in
315 infected cells (29), is required for efficient viral DNA packaging (1).
316 The formation of HSV replication compartments has been extensively
317 studied (14-17, 20, 23, 31). Several of the observations made in this report are
318 consistent with features of HSV replication compartments, including the
319 localization of the viral single-stranded DNA binding protein within replication
320 compartments and the dispersal of viral proteins from replication compartments
321 in the presence of an inhibitor of viral DNA synthesis (13, 23). However,
322 important features of HSV compartments differ from the features of the HCMV
323 replication compartments we describe here. BrdU staining of HSV DNA and
324 immunolocalization of several HSV proteins involved in viral DNA synthesis
325 indicate that viral DNA synthesis takes place throughout HSV replication
326 compartments (5, 6, 9, 14, 23), not at the periphery of compartments. Also, the

HSV counterpart of HCMV UL44, UL42, has yet to be observed concentrated at the periphery of replication compartments. To date UL42 has only been observed diffusely distributed throughout HSV replication compartments (6, 9), although it is unclear what form of UL42 the antibodies used in those studies recognize. Thus, the structure of HSV and HCMV replication compartments differ. Recently it has been demonstrated that HSV replication compartments coalesce at and reorganize nuclear speckles to enhance late viral mRNA export (4). The differences between HSV and HCMV replication compartment organization that we observe here may reflect differences in the requirement of each virus to utilize sub-nuclear structures during virus replication.

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456 **FIGURE LEGENDS**

457

458 **Figure 1 Localization of UL44 and host cell chromatin in infected cells.** (A)
459 HFF cells were infected at an MOI of 3, fixed at the indicated time points and
460 stained for IF analysis by deconvolution microscopy with Hoechst reagent
461 (shown in green) and MAb αICP36 recognizing UL44. αICP36 was detected by a
462 secondary antibody conjugated to a red fluorophore. Images were obtained by
463 acquiring sequential optical planes in the z-axis and deconvolved to remove out-
464 of-focus light from individual planes. A single deconvolved focal plane is shown in
465 each panel. Hoechst staining (green) is shown in the left hand column and UL44
466 staining (red) in the middle column. Panels in the right column show the images
467 in the right and middle columns merged. Arrows indicate areas of chromatin
468 marginalization. (B) The number of compartments containing UL44 in infected
469 cells. At each time point the number of compartments in 100 cells were
470 determined. The number of compartments at 48 h.p.i. and 96 h.p.i. are indicated
471 by grey and black bars, respectively.

472

473 **Figure 2 Detection by IF of UL44 and FLAG tagged UL44 in HCMV infected**
474 **cells.** Uninfected HFF cells (Figs. A, B and C) or HFF cells were infected at an
475 MOI of 3 with either HCMV strain AD169 (Figs. D and E) or HCMV-FLAG44 (28)
476 (Fig. F) (fixed 96 h.p.i.) were prepared for IF analysis by spinning disk confocal
477 microscopy by staining with MAb recognizing UL44 (MAbs 28-21 (A and D) and
478 CH16 (B and E)) or FLAG (anti-FLAG rabbit polyclonal Ab (C and F)). Abs were

479 detected using secondary antibodies conjugated to a red fluorophore. 3D images
480 were acquired by acquiring sequential optical planes in the z-axis using spinning
481 disk confocal microscopy. Each panel shows the merged XZ and YZ images
482 from each focal plane in the 3D stack acquired during microscopy and a single
483 confocal plane in XY. The antibody used in each case is noted above each
484 column. The time points analyzed are indicated to the left of the figure. The arrow
485 seen in panel E indicates an area where UL44 staining by MAb CH16 could not
486 be observed within the interior of the replication compartment.
487
488 **Figure 3 Analysis of UL44 and UL57 localization in infected cells.** HFF cells
489 were infected at an MOI of 3 with HCMV-FLAG, fixed at different time points and
490 stained for IF analysis using a polyclonal antibody recognizing FLAG and MAb
491 recognizing UL57and analyzed by spinning disk confocal microscopy. Panels in
492 the left column shows cells stained with Ab recognizing FLAG and a secondary
493 antibody conjugated to a red fluorophore. Panels in the middle column show cells
494 stained with Ab recognizing UL57 and a secondary antibody conjugated to a
495 green fluorophore. Panels in the right column show the images in the right and
496 middle columns merged. The white box in panel (vi) is a magnified view of the
497 indicated area.
498
499 **Figure 4 Visualization of EdU labeled viral DNA in infected cells.** (A) HFF
500 cells were infected at an MOI of 3 with AD169, fixed at the indicated time points
501 after 60 minutes incubation with EdU and stained for IF analysis using MAb

αICP36 recognizing UL44 and treated to detect EdU incorporated into DNA with a green fluorophore. Cells were analyzed by spinning disk confocal microscopy. Images were obtained by acquiring and merging sequential optical planes in the z-axis. Panels in the left column shows cells stained with MAb recognizing UL44 and a secondary antibody conjugated to a red fluorophore. Panels in the middle column show cells treated with a green florescent azide to detect EdU. Panels in the right column show the images in the right and middle columns merged. Each panel shows the merged XZ and YZ images from each focal plane in the 3D stack acquired during microscopy and a single confocal plane in XY. An area where neither UL44 nor Edu labeled DNA could be detected is indicated with an arrow in panel (vi). The white box in panel 2(vi) is a magnified image of the area indicated in the panel. The white box in panel (vi) is a magnified view of the indicated area. (B) Cells were pulsed with EdU for 30 mins (Fig. 4B(i)) and then incubated with thymidine for 30 mins in the absence of EdU (Fig. 4B(ii)). Infected cells were prepared as in Fig. 4A. Each panel shows the merged signals from the red (UL44) and green (EdU) fluorophores. Each image shows a single focal plane from the z-axis of the cells analyzed.

Figure 5 Localization of UL44 and EdU labeled DNA in the presence of PFA. HFF cells were infected at an MOI of 3. At 48h.p.i. cells were incubated with EdU for 60mins and fixed ((i)-(iii)) or then treated with phosphoformic acid (PFA) in the presence of EdU. Cells were fixed at the indicated time points after PFA treatment ((iv)-(xii)), then PFA was washed out of cells and cells incubated to

525 further time points in the presence of EdU ((xiii)-(xxi)). Cells were stained for IF
526 analysis using MAb αICP36 recognizing UL44 and treated to detect EdU
527 incorporated into DNA with a green fluorophore. Cells were analyzed by spinning
528 disk confocal microscopy. Panels in the left column shows cells stained with MAb
529 recognizing UL44 and a secondary antibody conjugated to a red fluorophore.
530 Panels in the middle column show cells treated with a green florescent azide to
531 detect EdU. Panels in the right column show the images in the right and middle
532 columns merged. All cells were imaged using the same magnification, but
533 because they are imaged using different focal planes, they may appear to be
534 different sizes.
535

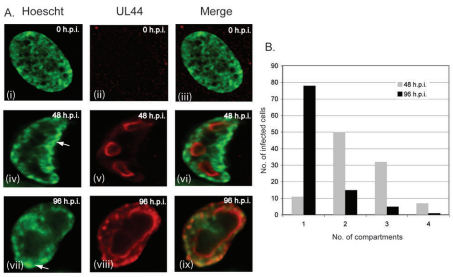

Figure 1

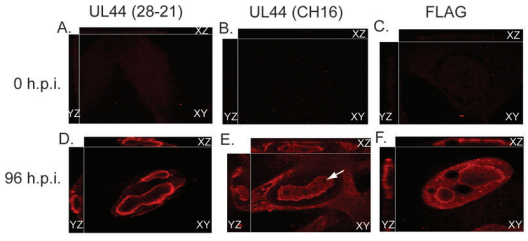

Figure 2

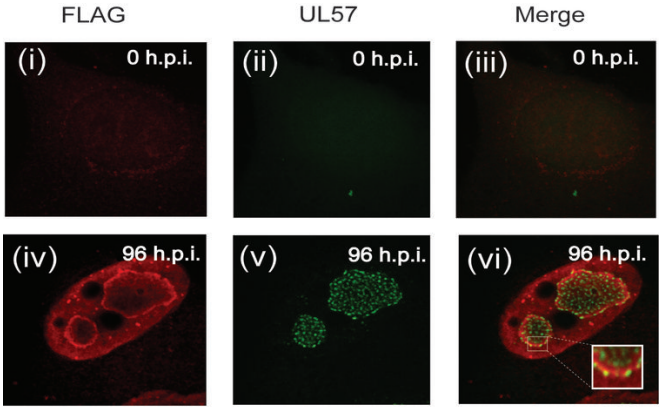

Figure 3

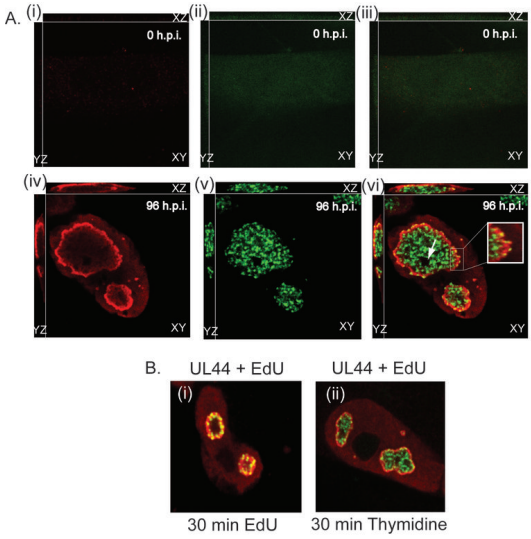

Figure 4

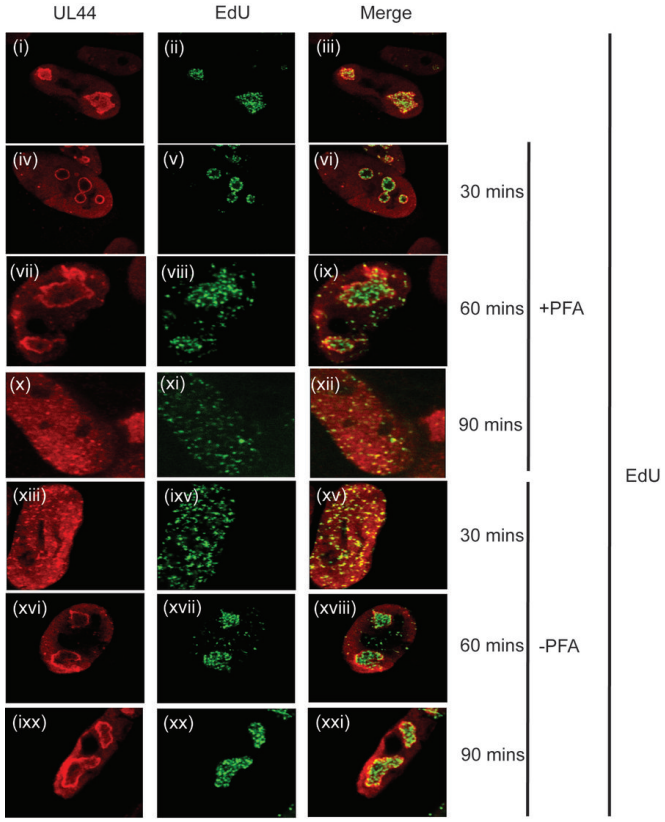

Figure 5